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14. ABSTRACT
We found that CDCP1 expression is decreased at the plasma membrane in invasive prostate cancers. This suggested that loss of CDCP1 may disrupt a normal epithelial cell function that contributes to a cancerous phenotype. In order to identify this hypotheltical normal cell function, required that we work with normal epithelial cells not immortal cell lines. This realization has caused a delay in work on Aim 3. We are pleased with our decision because it led to the identification of a novel signaling pathway GPCRs-SFK-CDCP1-PKCδ and a novel cell function. Disruption of this pathway by knockdown of PKCδ results in profound increases in SFK-mediated membrane protrusions. Membrane protrusions, in various forms including podosomes and invadopodia, are causal in invasion of cancer cells and remodeling of extracellular matrix. We are now determining if CDCP1 participates in this regulatory pathway through its interactions with PKCδ and SFKs. We suggest that Gp140 restricts PKCδ and SFK to the lateral and apical cell membrane and this suppresses membrane protrusions when GPCRs are activated. It is reasonable that the increased membrane protrusions may contribute to the loss of LM332 and hemidesmosomes in the basement membrane zone in prostate cancers. Our future research efforts will focus this new function for CDCP1 as a regulator of SFKs, PKCδ and membrane protrusions.

15. SUBJECT TERMS
Prostate cancer, Gp140/CUB Domain Containing Protein1, cell adhesion, invasion, metastasis, Laminin 332/5, integrins, membrane protrusions

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Final Comprehensive Annual Report for DOD Award W81XWH-08-1-0269
Carter Lab, FHCRC
(May 1, 2008 to April 30, 2012)

INTRODUCTION: SUBJECT, PURPOSE, SCOPE, BACKGROUND, ACCOMPLISHMENTS

1. Original Funded Hypothesis/Objective/Specific Aims: The central hypothesis of this project is that loss of Gp140/CDCP1 expression in prostate cancer promotes invasion and metastases. To test this hypothesis we propose in:

Specific Aim 1: To determine the role of Gp140 in adhesion, invasion and phosphorylation of the androgen receptor (AR). We will test the hypothesis that sustained surface expression of Gp140 prevents invasion of prostate cancer cells and limits the SFK-dependent activation of the AR.

Specific Aim 2: To test whether the expression level of Gp140 and of a Gp140 biomarker panel predicts the development of prostate cancer metastasis. We will test the hypothesis that the loss of Gp140 and E-cadherin expression and activation of SFKs are associated with prostate cancer metastasis in a case control study. The study population consists of men with high-grade prostate cancers (Gleason Sum 8-10) who relapsed in less than two years or did not recur for at least 5-years after treatment. In a logistic regression model, we will test the association of the Gp140 marker panel and prostate cancer recurrence.

Specific Aim 3: To determine whether inhibition of Gp140 with mAbs prevents metastases of PC3-GFP xenografts. We will test the hypothesis that inhibiting phosphorylation and internalization of Gp140 reduces invasion and metastasis of PC3-GFP xenografts. We will inhibit Gp140 phosphorylation with (1) an inhibitory antibody, with (2) recombinant soluble CUB domain and with (3) the cholesterol lowering agents Lovastatin. The response to treatment in mice *in vivo* will be followed by fluorescent-life imaging with the Xenogen IVIS imaging system and by expression analysis of the Gp140 marker panel.

2. Background and purpose: CDCP1, LM332 and integrin $\beta4$ are lost in invasive prostate cancer. Our research in the prior budget years (May 1, 2008 to April 31, 2011) established key findings that are necessary for our overall goal to understand if and how loss of Gp140/CDCP1 expression in prostate epithelium (**Fig. 1 below**) promotes invasion and/or metastases of prostate cancer cells. The decreases in CDCP1 at the plasma membrane of invasive prostate cancers correlates with loss of the basement membrane adhesive proteins LM332 and integrins $\alpha6\beta4$ and other components of the hemidesmosome cell junctions[1]. In contrast there are only minor alterations in other adhesion components including $\beta1$ integrins that mediate epithelial motility associated with invasive cancer. The loss of hemidesmosome components is frequently attributed to loss of basal cells in prostate cancer due to androgen driven differentiation and/or catabolism of the BM zone by proteases. However, our results suggest the loss of CDCP1 as a regulator of SFKs and PKCδ may also inhibit adhesion components of the BMZ. Research in the Carter Lab during this funding period (May 1, 2011 to April 31, 2012) has focused on four aspects of our overall goal to understand the function of CDCP1 in normal and cancerous epithelium.

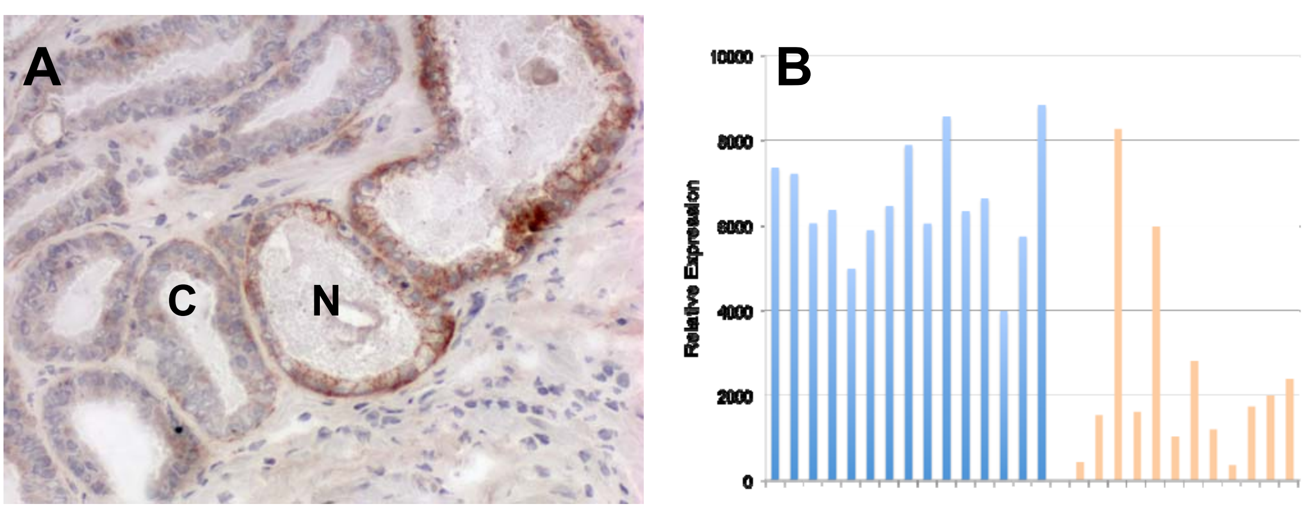
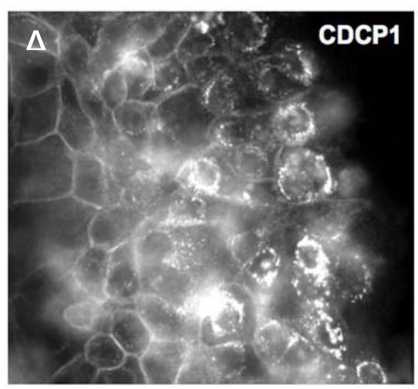


Fig. 1. CDCP1 expression at the protein level is decreased at the plasma membranes of invasive prostate cancers. (A) Staining of CDCP1 in the plasma membrane of normal (N) and cancerous (C) prostate glands. (B)Relative expression of CDCP1 in the plasma membrane of normal (blue) and cancerous (pink) prostate glands. We hypothesize that loss of CDCP1 alters the subcellular localization of SFKs and PKCδ contributing to increased invasion of prostate cancer epithelium.

BODY: RESEARCH ACCOMPLISHMENTS

Our finding that CDCP1 expression is decreased in invasive prostate cancers suggested that our future research should focus on the regulatory function of CDCP1 in normal epithelial cells. Therefore for most of the studies outlined below, we utilized primary cultures of normal human epidermal keratinocytes (HKs) in addition to immortal prostate and breast epithelial cell lines. This required us to delay planned work on Aim 3. Our overall research goal has generated progress in four areas relating to the function of CDCP1 in normal epithelium: Initial Activation of CDCP1 that generates outside-in Signals that result in downstream cell Function. Lastly, we are currently evaluating how loss of CDCP1 function in prostate epithelium may contribute to the pathology of prostate cancer. Progress in each of our four sub-goals are outlined below:

1. Initial Activation of CDCP1 by serum and GPCRs ligands: We have sought to identify biologically relevant compounds/ligands that can signal through CDCP1 and are relevant to prostate cancer. Our work focused on CDCP1 in normal cells to better understand what is lost in cancer. As a basis these studies, we previously reported that binding of anti-CDCP1 mAb to extracellular CDCP1 assembles CDCP1 with SFK(s) and Protein Kinase C delta (PKCδ) and promotes phosphorylation of all three components by SFKs [2, 3](**Fig.2 below**). However, we also had hints at more biologically relevant activator(s) of CDCP1 then the mAb: We previously reported that proteolytic cleavage of CDCP1/Gp140 to an 80 kDa transmembrane product initiates then prolongs phosphorylation of CDCP1 by SFKs [3, 4]. Further, we observed that serum as a component of epidermal wounds elevates expression, phosphorylation, proteolytic cleavage and internalization of CDCP1 at the protein level in models of epidermal wounds in vitro (**Fig. 2**).



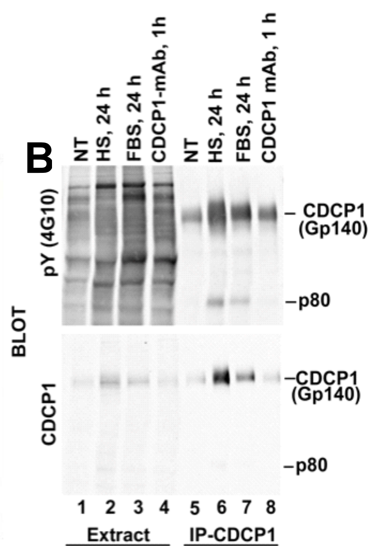
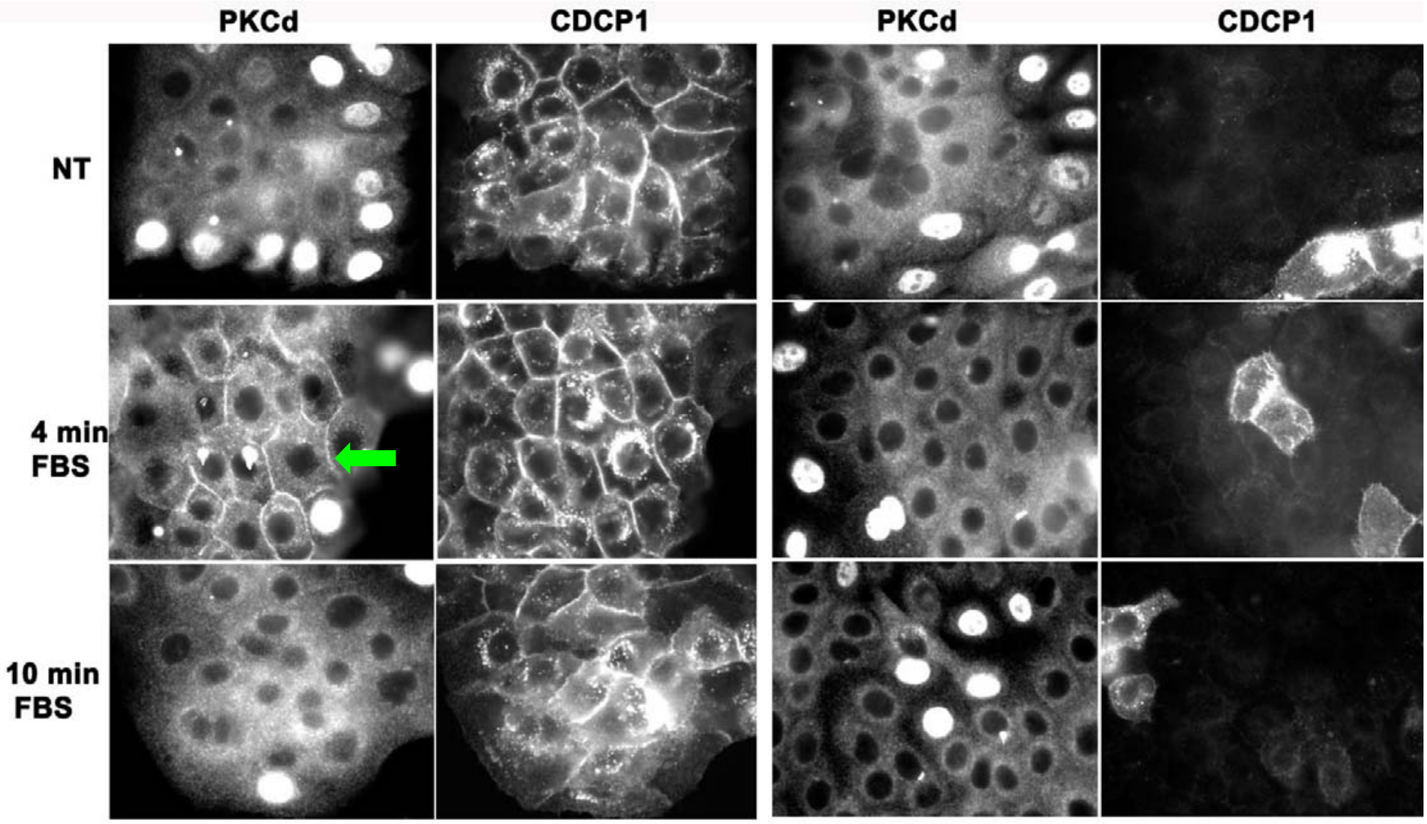


Fig. 2. (A) Serum addition to scrape wounds in keratinocytes promotes wound closure and increases expression and internalization of CDCP1 at the wound edge as detected by immunostaining with anti-CDCP1 mAb. (B) Expression and phosphorylation of CDCP1 are increased in keratinocytes by serum addition. CDCP1 was detected by immunoprecipitation (IP) with anti-CDCP1 Ab followed by immunoblotting with anti-CDCP1 Ab or anti-phosphotyrosine mAb (pY (4G10). HKs were grown in serum-free medum (NT, lane 1 and 5) or medium contain horse serum (5% HS, lanes 2 and 6; 24 hrs) or fetal bovine serum (5% FBS, lanes 3 and 7; 24 hrs) or anti-anti-CDCP1 mAb (1 hr).

Based on the results with serum or trypsin, we determined if serum regulates the subcellular localization of SFKs and PKCδ, similar to the activating anti-CDCP1 mAb. We found that FBS that elevates phosphorylation of CDCP1 can rapidly but transiently translocate PKCδ to the plasma membrane **(Fig. 3 below)**. Further, knockdown of CDCP1 or inhibition of SFKs with PP2 prevents the translocation of PKCδ to the plasma membrane. We conclude that one or more components of serum signals through CDCP1 to control the subcellular localization of PKCδ, and phosphorylation of CDCP1.

Fig. 3. Activation of keratinocytes with FBS transiently translocates PKCδ to the plasma membrane and cell-cell contacts (green arrow) in a CDCP1-dependent manor. Keratinocytes transfected with RNAi Control (Left Panels) or CDCP1 Knockdown RNAi (Right Panel) were treated without (NT) or with FBS (10% v/v) for either 4 min or 10 min. Cell were then doubled stained for PKCδ and CDCP1. FBS treatment of Control, but not CDCP1 KD cells, translocates PKCδ to cell-cell contacts but only at 4 min (green arrow).

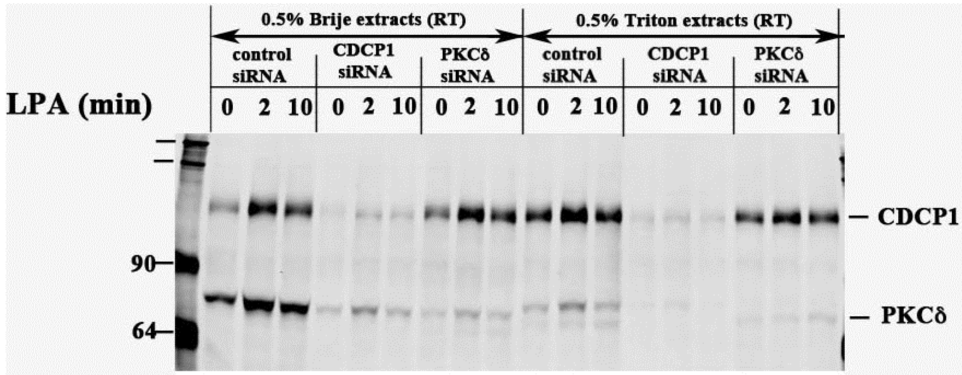


2. Outside-in Signaling through CDCP1: Serum has a major activating function in epidermal wounds. We hypothesized that phosphorylation of CDCP1 and translocation of PKCδ may be activated by one or more components of serum. For example, serum contains trypsin-like proteases that signal through the Protease Activated Receptors (PARs) in the GPCR family (Reviewed in [5]. We found that lysophosphatidic acid (LPA, 5 μM) or sphingosine 1 phosphate (SIP1, 2 μM) both present in serum can signal through their specific GPCRs to increase both expression and phosphorylation of CDCP1. Similarly, signaling through the PAR2 GPCR by trypsin or 2-Furoyl-LIGRLO peptide, or PAR1 by thrombin or SFLLRN peptide duplicates the activating effects of serum on phosphorylation and subcellular localization of CDCP1, SFK and PKCδ. Each of these signals through distinct GPCRs transiently increase phosphorylation of CDCP1 and membrane translocation of PKCδ. In general, ligand induced signals through GPCRs generate membrane extensions that participate in cell adhesion, migration and invasion. In control experiments, neither EGF nor Ca^{+2} (1 mM) addition caused significant increases in phosphorylation of CDCP1 or translocation of PKCδ suggesting that the signals through the GPCRs were selective in their effects on CDCP1 and PKCδ.

Next, we evaluated which signaling pathways and cell functions are activated through CDCP1. Within 1 min. of addition of LPA, Trypsin, Thrombin or SIP1 to HKs, PKCδ transiently translocates to the plasma membrane particularly at cell-cell contacts. The transient translocation last for 2-4 minutes and correlates with transient increases in phosphorylation of PKCδ, CDCP1 and SFK (**Fig. 4 below**). The translocation and phosphorylation of PKCδ is inhibited by knockdown of CDCP1 or PKCδ by RNAi or by inhibitors of SFKs (PP2 or SU6656). For comparison ligation of extracellular CDCP1 with antibodies generates a prolonged phosphorylation of CDCP1, PKCδ and SFK with prolonged recruitment of PKCδ to the plasma membrane when compared to the effects of GPCR ligands,.

Fig. 4. LPA promotes transient phosphorylation of CDCP1 and PKCδ. Knockdown of CDCP1 prevents phosphorylation of PKCδpY311 by SFKs but knock of PKCδ does not inhibit phosphorylation of CDCP1pY707. Keratinocytes were treated with Control, CDCP1 or PKCδ inhibitory RNA oligos, then treated with LPA (5 μM; for 0, 2 or 10 min). The cells were extracted

sequentially with 0.5% Brig 98 then 0.5% Triton X100 detergents. Extracts were immunoblotted with antibody that specifically recognizes phosphorylated CDCP1pY707 and PKCδpY311 (VGlpY).



Signals through at least four different GPCRs transactivate CDCP1 to recruit PKCδ to the plasma membrane. Therefore, we began a search for possible substrates for PKCδ and cell functions that may be regulated by the GPCR-SFK-CDCP1-PKCδ cascade. A number of known substrates for PKCδ have been published including Gap43, an actin- and PIP2-binding protein that regulates membrane protrusions in neuronal cells. Significantly, we found that binding of the activating antibody to CDCP1 that recruits PKCδ also recruits Gap43 to the CDCP1 cluster. KD of PKCδ does not inhibit recruitment of Gap43 to the CDCP1 cluster suggesting that active PKCδ was not required for interactions between Gap43 and CDCP1. However, signals through GPCRs did not recruit Gap43 to cell-cell contacts as it does for PKCδ. This suggested that Gap43 responds to CDCP1 independent of PKCδ.

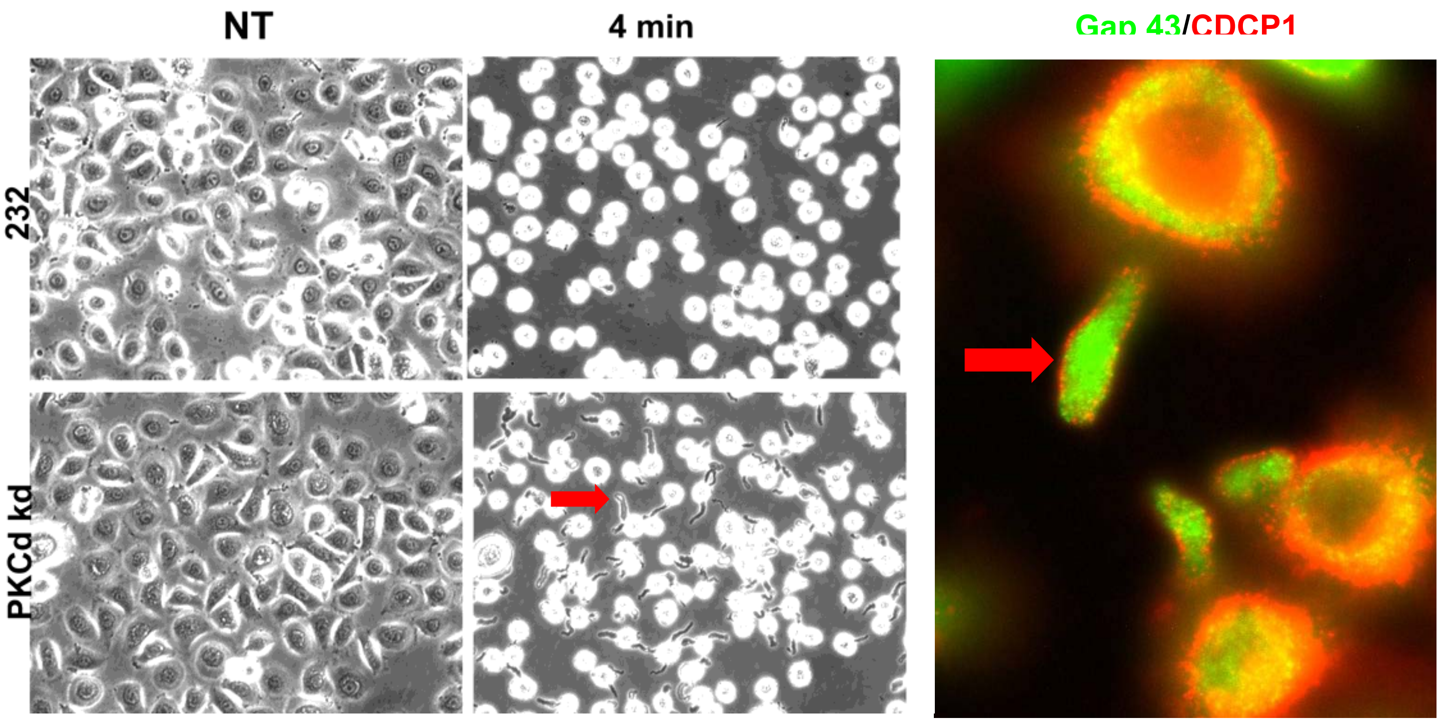


Fig. 5. Knockdown of PKCδ generates membrane protrusions ("Pinocchios"; red arrows) in keratinocytes when treated with Trypsin (Left Panels) or LPA (Right Panel) . (Left Panels) Keratinocytes with knockdown of PKCδ (PKCδ kd) or knockdown of CDCP1 (232) were treated without (NT) or with trypsin (0.05% for 2 min→ chase 2 min = 4 min) causing cell rounding. Significantly, the PKCδ KD cells, but not CDCP1 KD cells or Control cells (not shown) extended pinocchios detected by phase microscopy. (Right Panel) LPA treatment of PKCδ KD keratinocytes generates Pinocchios that stain strongly for CDCP1 (red; red arrow) at the plasma membrane and Gap 43 (green).

3. A Function for PKCδ, CDCP1, and SFKs in GPCR mediated membrane protrusion: Ligand-induced signals through GPCRs promote membrane extensions that contribute to cell adhesion, spreading, migration and invasion [5]. In addition these ligands also signal through CDCP1 to translocate PKCδ to the plasma membrane. We hypothesized that CDCP1, SFK and PKCδ may participate as either a positive or negative regulator of the membrane extension. Knockdown of either CDCP1 or PKCδ or inhibition of SFKs prevents translocation of PKCδ to the plasma membrane in response GPCR ligands. Significantly, LPA, serum or trypsin activation of PKCδ KD cells causes the suprising assembly of prominent membrane protrusions, here termed "pinocchios" (**Fig. 5 above**). The pinocchios are generated concurrent with the phosphorylation of SFKs and CDCP1 in response to LPA or serum. The pinocchios are prevented by inhibition of SFKs with PP2 or inhibition of myosin-mediated cell contraction with blebbistatin. Consistently, myosin light chain (MLC) is phosphorylated on S19 by MLCkinase or Rho Kinase (ROCK) and co-localizes with phosphorylated CDCP1 and SFK in the pinocchios. Together these results suggest that signals through GPCR that generate membrane extensions are limited/regulated by PKCδ to prevent the membrane herniation. We are currently determining if CDCP1-PKCδ interactions at the plasma member regulate PKCδ to prevent the pinocchios. We are also determining if the membrane pinocchios contribute to changes in the BMZ described prostate cancer.

4. Impact in Cancer: Membrane protrusions, in contrast to lamellipodia extension, are suggested to generate abnormal cell invasion in cancers (reviewed in [6]). Knockdown of PKCδ in mice leads to lethal inflammatory defects and increased sensitivity to TPA tumor promotion (Reviewed in [7]). This suggests that biological function for PKCδ is to inhibit excessive flammation and tumor promotion. Based on this concept, we hypothesize that CDCP1-dependent localization of PKCδ to the plasma membrane in response to serum, proteases, LPA or SIP1 may limit inflammation and tumor promotion. We hypothesize that interaction of CDCP1 with PKCδ in response to GPCR activation prevents membrane protrusions that contribute to cell invasion. We are currently evaluating the effects of knockdown of CDCP1 and PKCd in cell invasion assays in collagen and matrigel in response to activation of GPCRs.

SUMMARY OF KEY RESEARCH ACCOMPLISHMENTS FOR EACH FUNDED PERIOD

Period: May 1, 2008 to April 31, 2009.

1. Yeast signal outside-in through Gp140, SFKs and PKCδ to increase migration and cell-cell adhesion of keratinocytes and other epithelial cells for the purposes of exclusion of microbes from epithelial tissues.
2. ActGp140 mAbs stimulates host defense mechanisms and therefore may be efficacious in reducing invasion of pathogens into tissue.
3. Stress or inflammatory stimuli in the prostate may lead to phosphorylation and internalization of Gp140 as and early event in the progression of prostate cancer and may have implication for invasion and metastasis of prostate cancer cells.
4. ActGp140 mAb signals through Gp140 to selectively inhibit cell-substrate adhesion of PC3 cells on mesenchymal ECM but not laminin 332. Because prostate cancer cells lose laminin 332, we suggest that ActGp140 mAbs alone or in conjunction with anti-integrin mAbs may be of value in selectively disrupting adhesion of cancer cells to mesenchymal ECM but not normal cells that synthesize and adhere to laminin 332.
5. Staining of normal prostate and prostate cancers suggests that Gp140, LM 332 and integrin β4 are lost from prostate cancers. Further, Gp140 and integrin β4 are lost prior to LM 332. This suggests that anti-Gp140 mAbs may have value in detecting early changes in prostate cancer progression.

Period: May 1, 2009 to April 31, 20011.

1. Successfully established the technology for transient or stable knockdown of mRNA encoding Gp140/CDCP1 in multiples cell types (keratinocytes, PC3, PECs, MCF10A and HS5). This was necessary for Specific Aims 1 and 3.
2. In collaboration with the Beatrice Knudsen Lab, we found that localization of Gp140 to the plasma membrane of prostate epithelial cells is decreased or lost in PIN, invasive prostate cancer and metastatic prostate cancers when compared to normal prostate epithelium. In contrast, cytoplasmic levels of Gp140 are sometimes elevated or not lost in metastatic prostate cancer. Therefore the decreases in the membrane form of Gp140 occurs prior to loss of integrin β4 or LM332 in prostate cancer.
3. We found that knockdown of Gp140 causes decrease in integrin β4, E-cadherin and syndecan1 at both the protein and mRNA levels. In culture, the knockdown of Gp140 causes a reduction in assembly of hemidesmosome and adherens junction. These results suggest that the decreases in Gp140 observed in PIN may participate or cause the loss of integrin b4 and hemidesmosomes in invasive prostate cancer.

Period: May 1, 2011 to April 31, 2012.
1. Identification of serum and multiple GPCR ligands in serum (eg. LPA, SIP1, Trypsin and Thrombin) as transactivators of CDCP1. This observations was critical for the subsequent identification of PKCδ as a regulator of epithelial cell membrane extension required for cell adhesion and motility.
2. Identification that multiple GPCR ligands can promote the transient translocation of PKCδ to the plasma membrane and cell-cell contacts of epithelial cells. The translocation of PKCδ is dependent on both CDCP1 and SFKs.
3. Identification of Gap43 as a component of epithelial cell membrane protrusion and a possible interactive component for CDCP1.
4. Identification of a novel function for the GPCR-SFK-CDCP1-PKCδ communication pathway in restricting membrane protrusions in response to GPCR signaling.

REPORTABLE OUTCOMES

Presentations:
Gordon Research Conference: "Epithelial Differentiation & Keratinization." Mount Snow Resort, West Dover, VT, July 3-8, 2011. CDCP1 regulates epithelial cell adhesion and polarity in response to yeast. Tatiana Zaitsevskaia, Robert Sullivan, Tod Brown, Madeline Wilson. Theodore White and William G. Carter.
FASEB Summer Research Conference: "Signal transduction through tetraspanins and other multi-protein cell surface complexes." June 22-27, 2008, New Haven, Connecticut. Lecture by William Carter, entitled: "Microbes signal through Gp140/CDCP1 in epithelial cells to regulate src family kinase(s) and protein kinase c delta (PKCδ) leading to changes in cell-cell and cell-substrate adhesion."
Patents: U.S. Provisional Application No. 60/954,177; Filed August 6, 2007, Modulation of Cell Junctions, Inventor(s): Carter.
Manuscripts: Three different manuscripts are currently being assembled based on the results presented in this and past DOD Progress Reports.

CONCLUSIONS

We found that CDCP1 expression is decreased at the plasma membrane in invasive prostate cancers. This suggested that loss of CDCP1 may disrupt a normal epithelial cell function that contributes to a cancerous phenotype. In order to identify this hypothetical normal cell function, required that we work with normal epithelial cells not immortal cell lines. This realization has caused a delay in work on Aim 3. We are pleased with our decision because it led to the identification of a novel signaling pathway GPCRs-SFK-CDCP1-PKCδ and a novel cell function. Disruption of this pathway by knockdown of PKCδ results in profound increases in SFK-mediated membrane protrusions. Membrane protrusions, in various forms including podosomes and invadopodia, are causal in invasion of cancer cells and remodeling of extracellular matrix. We are now determining if CDCP1 participates in this regulatory pathway through its interactions with PKCδ and SFKs. We suggest that Gp140 restricts PKCδ and SFK to the lateral and apical cell membrane and this suppresses membrane protrusions when GPCRs are activated. It is reasonable that the increased membrane protrusions may contribute to the loss of LM332 and hemidesmosomes in the basement membrane zone in prostate cancers. Our future research efforts will focus this new function for CDCP1 as a regulator of SFKs, PKCδ and membrane protrusions. We would like to thank the DOD for its investment in our research efforts.

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APPENDICES

None